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Identification of a domoic acid-producing *Pseudo-nitzschia* species (Bacillariophyceae) in the Dutch Wadden Sea with electron microscopy and molecular probes

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Biological monitoring in the Dutch Wadden Sea between November 1993 and July 1994 revealed *Pseudo-nitzschia*-like pennate chain-forming diatom species with cell numbers ranging from 10^2 to 10^5 per litre. Cultured isolates and field samples were examined by electron microscopy, which revealed the majority to be *Pseudo-nitzschia pungens*. This species dominated over other diatoms in the phytoplankton population during November 1993 and at the end of June 1994. At the beginning of June 1994, *P. fraudulenta* was also present; occasionally, *P. delicatissima* was observed. One isolate showed the characteristic morphology of *P. multiseries*. Species-specific polyclonal antibodies and large-subunit (LSU) rRNA-targeted oligonucleotides for North American strains of *P. multiseries* and *P. pungens* applied to the European isolates, confirmed species designations based on electron microscopy. The isolate of *P. multiseries* from the Dutch Wadden Sea produced domoic acid; after 55 days of growth about 19 pg per cell was measured. This is the first report of a domoic acid-producing *P. multiseries* isolated from European coastal waters.

Key words: diatoms, domoic acid, molecular probes, *Pseudo-nitzschia* spp., toxic algae

Introduction

Amnesic shellfish poisoning (ASP) was reported for the first time at Prince Edward Island (Canada) in 1987, where consumption of contaminated mussels resulted in illness of over a hundred people and the death of several of them (Perl *et al.*, 1990). ASP results in gastrointestinal and neurological disorders, a temporal loss of memory and, in more severe cases, coma and death ensue (Perl *et al.*, 1990; Todd, 1993). The toxin responsible for this syndrome has been identified as domoic acid (DA), a neuroexcitatory amino acid related to glutamate (Wright *et al.*, 1989).

Domoic acid has been found in red macroalgae (Takemoto & Daigo, 1960; Impellizzeri *et al.*, 1975) as well as in pennate diatoms of the genus *Pseudo-nitzschia* (Bates *et al.*, 1989; Lundholm *et al.*, 1994). *P. multiseries* (Grunow *ex* Cleve) Hasle (Hasle, 1995), which proved to be toxic, has been observed in North America, Japan, Argentina, Scandinavia (Hasle, 1976; Fryxell *et al.*, 1990; Hallegraeff, 1993; Walz *et al.*, 1994) and New Zealand (D. M. Anderson & N. Towers, personal communication), often co-occurring with the non-toxic species *P. pungens* (formerly known as *P. pungens* (Grunow) Hasle forma *pungens* (Hasle) Hasle). Other species of the *Pseudo-nitzschia* complex have also been implicated in the production of DA. *P. australis* Frenguelli (= *P. pseudoseriata* Hasle) was responsible for the death of cormorants and pelicans feeding on contaminated anchovies on the west coast of North America (Buck *et al.*, 1992; Fritz *et al.*, 1992; Garrison *et al.*, 1992). Another species, *P. pseudodelicatissima* (Hasle) Hasle, has been linked to toxic events in the Bay of Fundy, eastern Canada (Martin *et al.*, 1990), and has also been found throughout western Europe where it bloomed along the coast of Denmark in 1992 (Lundholm *et al.*, 1994) and in Kiel Bay, western Baltic Sea (Hansen & Horstmann, 1993). Although this species is reported to produce domoic acid (Martin *et al.*, 1990), isolates from the bloom in Danish coastal waters were not toxic (Lundholm *et al.*, 1994). In western Europe another member of the complex, *P. seriata* (Cleve) H. Peragallo, can also produce the toxin (Lundholm *et al.*, 1994). *P. seriata* occurs in colder areas of the Northern Hemisphere and represents one of the main pennate diatom species in the North Atlantic (Hasle, 1976). Shimizu *et al.* (1989) and

Smith *et al.* (1991) reported toxin production by *P. delicatissima* (Cleve) Heiden and a member of another diatom genus, *Amphora coffeaeformis* (C. Agardh) Kützing.

The threat of domoic acid and ASP has been taken seriously in areas of shellfish production, resulting in an intensified study of and search for toxic *Pseudo-nitzschia* species. In addition to traditional electron microscopical observations of frustule morphology (Hasle, 1965, 1995), new molecular approaches allow species-specific identification, even when the alga is rare (Vrieling *et al.*, 1995). For both toxic *P. multiseries* and non-toxic *P. pungens*, species-specific polyclonal antibodies (Bates *et al.*, 1993) and oligonucleotide probes (C. A. Scholin, unpublished) were developed using North American strains as reference taxa. Until now they had not been tested on European isolates. In this paper, we describe the application of these probes to conspecific algae from the Dutch Wadden Sea and report on the capacity for domoic acid production by a European isolate of *P. multiseries*.

Materials and methods

Sampling and culturing

Samples for the Dutch monitoring programme were taken in the Dutch Wadden Sea (Fig. 1) between November 1993 and July 1994. Sampling was performed using Niskin bottles mounted on a rosette sampler. Live samples (1 l) were examined within 2 days in order to establish cultures. In addition, formalin (4·0% final concentration) and 0·4% (v/v) Lugol-fixed field samples were examined in case no isolates could be obtained. Crude isolates were obtained by microscopical selection of chains, which were washed with sterile seawater before transfer into F/2 enriched seawater (Guillard, 1975). After initial growth at 12°C, the cultures were cloned by selecting either single cells or small chains (typically of fewer than four cells). Clones were grown in F/2 enriched media at 16°C under a light/dark regime of 12 h light and 12 h dark at a photon irradiance of approximately 75 μmol m^{-2} s^{-1}.

Morphological analysis

For comparative studies of frustule morphology, various strains of *Pseudo-nitzschia* species were used (Table 1); a number of strains were obtained from the Provasoli-Guillard Culture Collection, West Boothbay Harbor, Maine (CCMP). Light microscopy of cultures and of live and fixed field samples was performed using an Olympus IMT2 inverted microscope. For electron microscopical analysis, cells of exponentially growing cultures or fixed field samples were concentrated by centrifugation (1000 rpm, 5 min), washed once with 1 M HCl, and treated with concentrated, boiling HNO_3 according to Boyle *et al.* (1984) to dissolve detritus and other organic matter. Cleaned frustules were washed twice with double-distilled water. For scanning electron microscopy, frustules were resuspended in 100% ethanol, mounted on stubs, air dried, and coated with gold before examination in a JEOL STM35. Transmission electron microscopy was performed on cleaned, ethanol-washed frustules, pipetted on Formvar-coated grids, which were air-dried and stained with 1% (w/v) uranyl acetate before examination using a Philips EM201 and CM10.

Immunofluorescence and oligonucleotide probing

Polyclonal antibodies specific for non-toxic *P. pungens* and toxic *P. multiseries* (Bates *et al.*, 1993) were applied using a 96-well vacuum-manifold system (Millipore, Milford, MA) following the immunofluorescence assay described by Vrieling *et al.* (1994). Both antibodies were used at a dilution of 1 : 50. Labelling of cultured cells was compared with negative controls (omitting primary antibodies or using normal serum) and positive controls (previously identified strains). Fluorescence was examined under a Zeiss Axioscope equipped with a LP 520 for simultaneous observation of green fluorescein isothiocyanate (FITC) and red chlorophyll fluorescence.

Oligonucleotide probing was performed as follows. Cells were fixed for 1 h at room temperature with freshly prepared paraformaldehyde (1% (w/v) final concentration). After fixation cells were resuspended in 100% MeOH to reduce chlorophyll fluorescence. Fixed and extracted cells were concentrated and washed three times with 500 μl hybridization buffer (0·1 M Tris (pH 7·8) containing 0·75 M NaCl, 5 mM EDTA (pH 8·0), 0·1% (v/v) NP40, 0·1% (v/v) poly dA), then resuspended in 150 μl hybridization buffer. Aliquots of 50 μl were loaded in 0·5 ml Eppendorf tubes and 3–5 μl of probe at 50 ng μl^{-1} was added before incubating for several hours at 55°C. Sequences of the *P. multiseries*- and *P. pungens*-specific probes, along with results of specificity testing are available elsewhere (Miller & Scholin, 1996). The plates were protected from bright light to maintain fluorescence stability of the probe. After incubation, 100 μl of pre-warmed (45°C) SET (4 mM Tris (pH 7·8); 3 mM NaCl; 0·2 mM EDTA (pH 8·0)) was added, cells were pelleted immediately, and the supernatant was removed. Subsequently, cells were resuspended in 150 μl pre-warmed SET and incubated for 3–5 min before they were pelleted again. As much supernatant as possible was removed and a drop of antifade (Slowfade; Molecular Probes, Oregon) was added before samples were examined for fluorescence.

Domoic acid analysis

Three species of *Pseudo-nitzschia* were used to determine the production of domoic acid. Two strains of *P. multiseries* (CCMP1573 as a control and the Dutch isolate W420Ppm5) and one strain of *P. pungens* (W420Ppp3) were grown in 500 ml F/2 enriched seawater under conditions described above. At 5–10 day intervals cell density was estimated by microscopical counting of at least 200 cells using a Sedgwick–Rafter chamber. In each sample 10^6 to 10^7 cells were concentrated by centrifugation

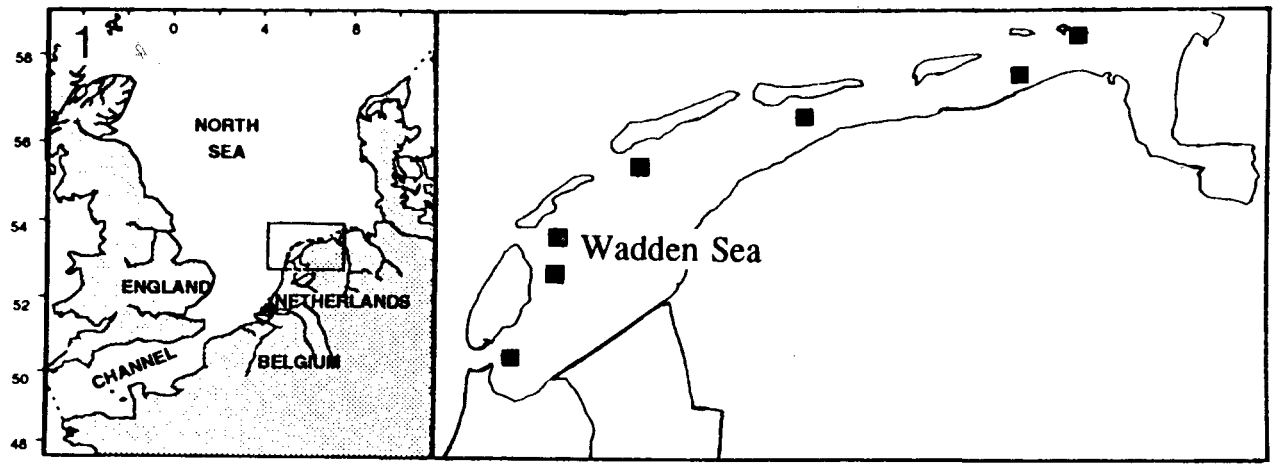


Fig. 1. Location of Dutch Wadden Sea and sample stations examined for presence of *Pseudo-nitzschia* species during November 1993 and July 1994. Stations, numbered from left to right: W30, WM/VL, O_1, W420, BVC6, W590, E250.

(2000 rpm, 10 min) and quickly washed in sterile seawater before the cell pellet was stored at −70°C. For analysis of domoic acid, cells were disrupted in 10% (v/v) acetonitrile (1·5 ml) for 10 min in an ultrasonic bath. After centrifugation (4000 rpm, 10 min), the extract was filtered through a 0·45 μm filter before use. Samples (10 μl) were analysed using a Spectra-Physics HPLC system (Spectra-Physics Analytical, Fremont, CA) composed of a SP8800 ternary HPLC pump, an SP8780 autosampler, and a Spectra FOCUS UV detector (absorption maximum 242 nm for domoic acid). The stationary phase (Nova-Pak C18 column, 4 μm, Millipore, Milford, MA) was eluted with acetonitrile and double-distilled water (10·5 : 89·5, pH 2·7 by adding orthophosphoric acid) with a flow speed of 1·2 ml min^{-1}. Analysis time was 20 min per sample. The detection limit of the system was 0·3 ng domoic acid. Additionally, a number of samples containing only culture medium taken from a presumably stationary *P. multiseries* (W420Ppm5) culture were analysed for domoic acid. In order to confirm the presence of domoic acid in the chromatograms, a number of samples were spiked (5 μl) with a standard solution of domoic acid (prepared from 90% pure domoic acid (Sigma, St Louis, MO), final concentration 100 mg l^{-1}) to filtered extracts (245 μl). The presence of domoic acid was confirmed in Germany (ICBM, Wilhelmshaven) using the FMOC method according to Pocklington *et al.* (1990) and a TLC method (Dallinga-Hannemann *et al.*, 1995).

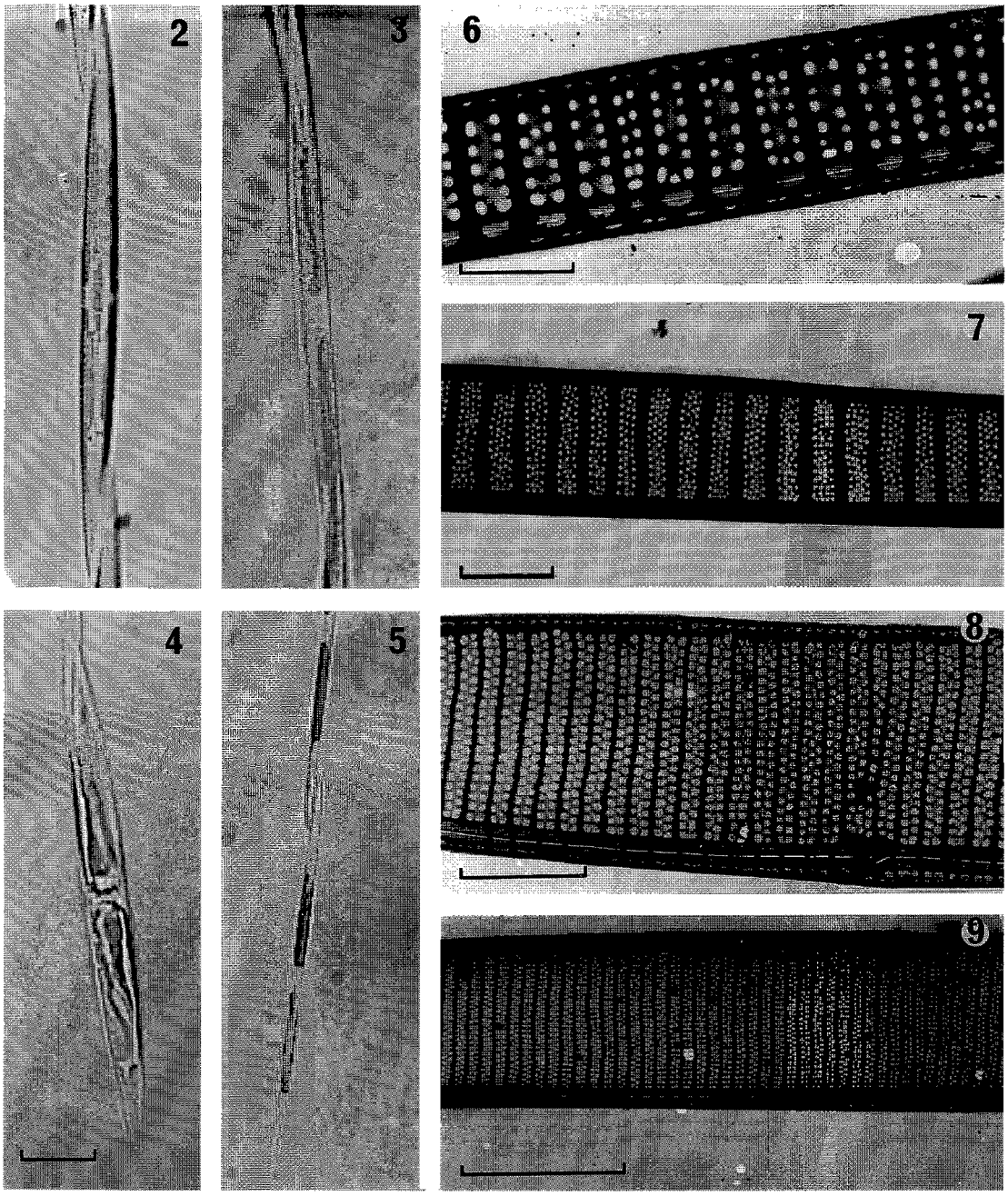
Table 1. The different *Pseudo-nitzschia* species examined in this study for verification of frustule morphology, reactivity in both immunolabelling and nucleotide probing, and toxicity

Species	Strain	Immunofluorescence		Oligonucleotide probing		Domoic acid producer	Reference
		Anti-*pungens*	Anti-*multiseries*	*pungens*	*multiseries*		
P. delicatissima (Cleve) Heiden	Field sample	−	−	n.d.	n.d.	n.d.	
P. fraudulenta Hasle	CCMP 1561	−	± (−)[a]	n.d.	n.d.	No	CCMP Catalogue
	Field sample	−	−	n.d.	n.d.	n.d.	
P. multiseries (Grunow *ex* Cleve) Hasle	CCMP 563	−	++	n.d.	n.d.	Yes	CCMP Catalogue
	CCMP 1573	−	++	n.d.	n.d.	Yes	CCMP Catalogue
	W420Ppm5	−	++	−	++	Yes	Present study
P. pungens (Hasle) Hasle	CCMP 1566	++	−	++	−	No	CCMP Catalogue
	W030Ppp1	++	−	++	−	No	Present study
	W420Ppp1	+	−	++	−	No	Present study
	W420Ppp2	+	−	++	−	No	Present study
	W420Ppp3	++	−	++	−	No	Present study
	W420Ppp4	+	−	++	−	No	Present study
	W590Ppp1–7	++	−	n.d.	n.d.	No	Present study
P. pseudodelicatissima (Hasle) Hasle	CCMP 1565	−	± (±)	n.d.	n.d.	?	
	LØGSTØRBRY	−	± (±)	n.d.	n.d.	No	Lundholm *et al.* (1994)
	EJBYHAVN4	−	± (−)	n.d.	n.d.	No	Lundholm *et al.* (1994)

n.d., not determined.

Fluorescence intensities are expressed as: ++, strong labelling; +, positive labelling; ±, weak labelling; −, no detectable labelling.

[a] Antibodies diluted 1 : 100 instead of 1 : 50.

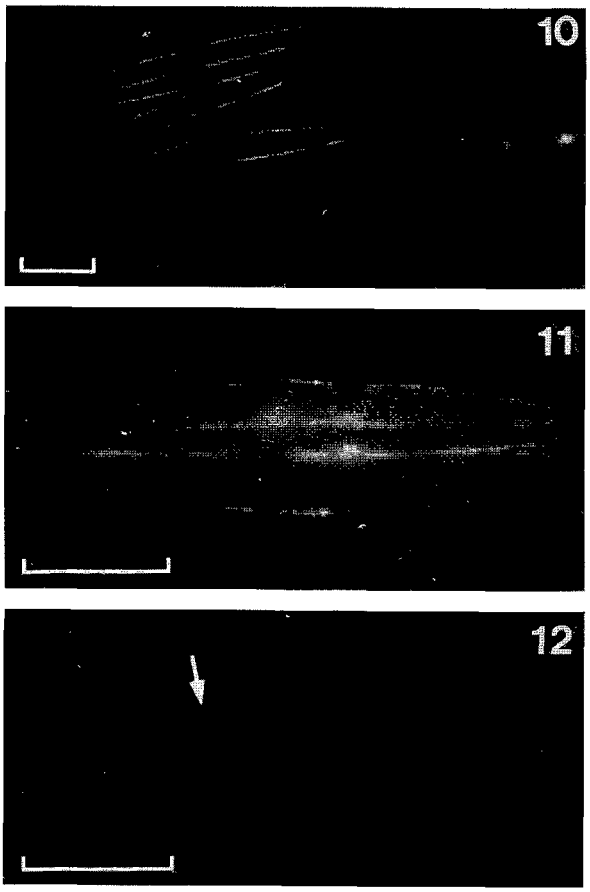


Figs 2–9. Phase-contrast light micrographs and electron micrographs of *Pseudo-nitzschia* species from the Dutch Wadden Sea. Figs 2, 6. *P. pungens*. Figs 3, 7. *P. multiseries*. Figs 4, 8. *P. fraudulenta*. Figs 5, 9. *P. delicatissima*. Scale bars represent: Figs 2–5, 10 μm; Figs 6–9, 2 μm.

Results

Pseudo-nitzschia species (10^3–10^4 cells l^{-1}) were observed between November 1993 and July 1994 at all stations in the Dutch Wadden Sea (Fig. 1). Although exact cell densities of individual species are unknown, the total number of *Pseudo-nitzschia* varied but never exceeded 10^4 cells l^{-1} before the end of June 1994. In samples taken on 27 June 1994, however, *P. pungens* dominated (over 90% of the pennates) in samples from just south of the Island of Terschelling (station O_1; Fig. 1).

Light microscopy of live samples and cultures indicated that cells were connected to each other in chains of 2 to 12 cells (Figs 2–5). Cell sizes of different isolates were 65–120 μm in length and 3·5–10·0 μm in width. Light and electron microscopical analysis revealed morphological characteristics of taxonomic importance: the density of striae per 10 μm and the pores (number of rows and size) per stria (Figs 6–9). All isolates of *P. pungens* had two rows of pores on each stria and about 10 striae per 10 μm (Figs 2, 6). *P. multiseries* differs from *P. pungens* by having at least three rows of smaller pores (Figs 3, 7). *P. fraudulenta* (Figs 4, 8) has a different type of pore, which is wider and has clusters of smaller pores in the basal silica layer (not shown). Besides the difference in pore type, *P. fraudulenta* has wider cells than other species: 6–10 μm instead of 2–6·5 μm. A smaller species, *P. delicatissima*, was easily identified by its cell size:



Figs 10–12. Fluorescence microscopy of immunolabelled cells of *Pseudo-nitzschia* spp. Fig. 10. *P. pungens* labelled using anti-*P. pungens* antibodies. Fig. 11. *P. multiseries* labelled using anti-*P. multiseries* antibodies. Fig. 12. *P. multiseries* labelled with anti-*P. pungens* antibodies; the arrow indicates the position of chlorophyll autofluorescence. Scale bars represent 25 μm.

1·5–2·5 μm in width and 45–60 μm in length (Figs 5, 9). Over the period of examination, *P. pseudodelicatissima* was not observed in any sample.

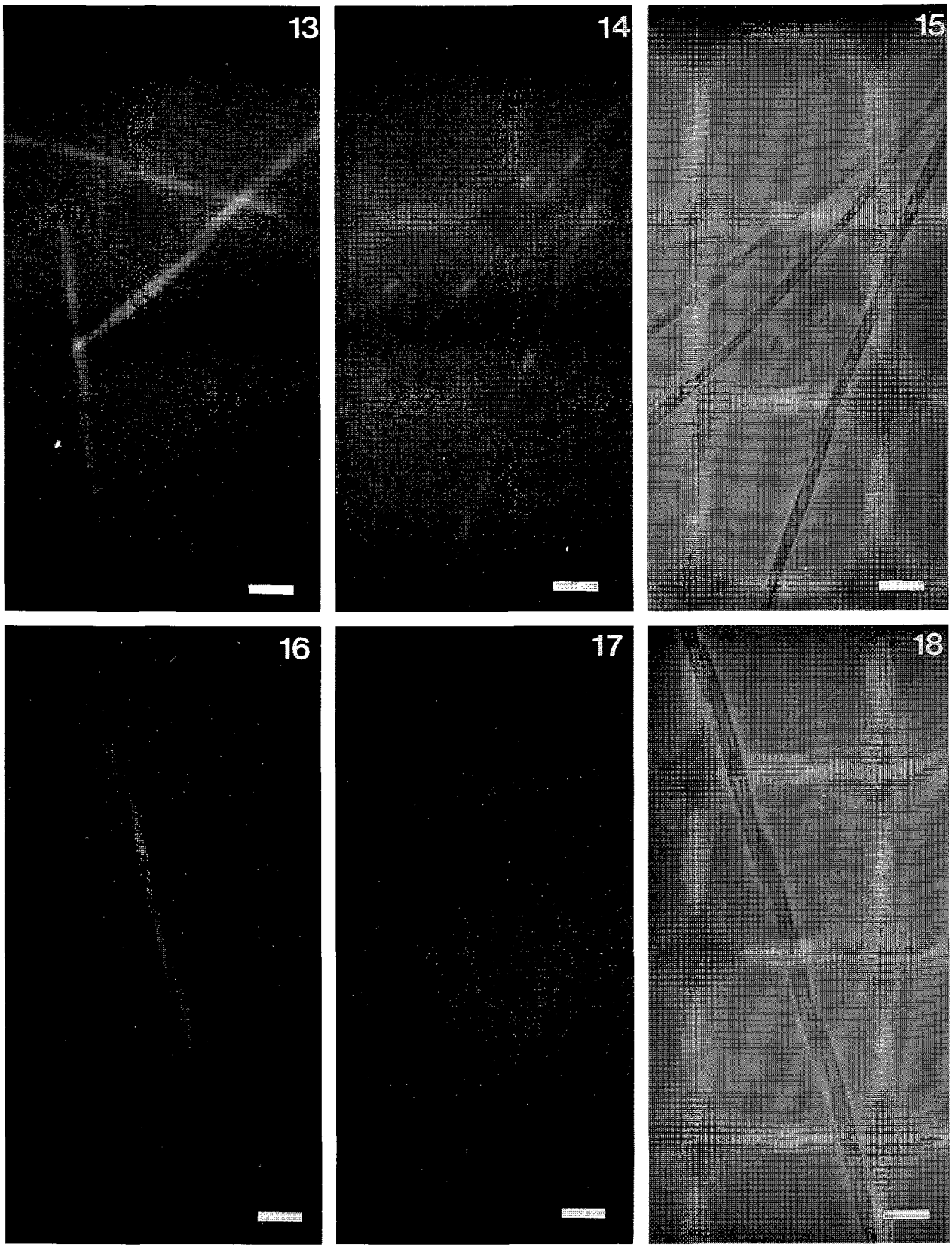
The identification of *P. pungens* and *P. multiseries* was confirmed using both species-specific antibodies and oligonucleotide probes prepared for North American isolates of these species. The polyclonal antibody raised against *P. pungens* reacted exclusively with isolates of *P. pungens* (Table 1) and showed a bright fluorescence signal at the outer cell surface (Fig. 10). The polyclonal serum raised against *P. multiseries* reacted specifically with the *P. multiseries* isolate W420Ppm5 and revealed a similar bright fluorescence at the outer cell surface (Fig. 11). The fluorescence of labelled cells was compared with cross-species controls in which only chlorophyll fluorescence was observed (Fig. 12). Slight labelling, however, could be observed at the cell surface of both *P. pseudodelicatissima* and *P. fraudulenta* when the anti-*P. multiseries* antibody was applied (Table 1). The labelling intensity of these cells was significantly lower than that of *P. multiseries* cells, whereas with lower antibody concentrations no significant labelling could be observed for *P. fraudulenta* (Table 1). Species-specific oligonucleotide probes gave comparable results. Here, the fluorescence was located inside the cell instead of at the outer cell surface (Figs 13–18). Isolates of *P. pungens* reacted with the *P. pungens*-specific probe (Table 1, Fig. 16) whereas the isolates of *P. multiseries* reacted only with the *P. multiseries*-specific probe (Table 1, Fig. 13). No cross-reaction was observed when either *P. pungens* or *P. multiseries* was probed with probes specific to the other species (Figs 14, 17).

Toxicity of *P. multiseries* (W420Ppm5) isolated from the Wadden Sea was compared with a known toxic strain of this species (CCMP1573). Growth was followed for 55 days, while the same samples were analysed for the presence of domoic acid. For both strains of *P. multiseries*, the stationary phase was entered at day 27, whereas for *P. pungens* (W420Ppp3), the negative control, stationary growth started at day 33 (Fig. 19). During the stationary phase, domoic acid was detected in both strains of *P. multiseries* but not in *P. pungens* (Fig. 19). Remarkably, the production of domoic acid in CCMP1573 was below the detection limit after day 33, while the Wadden Sea isolate showed an increase in toxin production to 18·99 pg domoic acid per cell at day 55 (Fig. 19). As expected, no domoic acid could be detected in *P. pungens* (W420Ppp3) even following extraction of over 10^7 cells at days 40 and 55 (Fig. 19). In the late stationary phase, traces of domoic acid were measurable in culture media of the Wadden Sea isolate (not shown). The peak of domoic acid appeared at a retention time of 5·8 min (Fig. 20).

Discussion

Between November 1993 and July 1994 the following *Pseudo-nitzschia* species were identified in the Dutch Wadden Sea: *P. pungens*, *P. multiseries* (formerly *P. pungens* Grunow forma *multiseries* (Grunow *ex* Cleve) Hasle), *P. fraudulenta*, and *P. delicatissima*. *P. pseudodelicatissima*, which bloomed in 1992 in Danish coastal waters and Kiel Bay, western Baltic Sea (Hansen & Horstmann, 1993; Lundholm *et al.*, 1994), was not observed. The full distribution pattern of *Pseudo-nitzschia* species cannot yet be presented due to a lack of proper species identifications in other parts of Dutch coastal waters.

Studies of domoic acid production by European strains of *Pseudo-nitzschia* species were undertaken only recently (Lundholm *et al.*, 1994). Isolates of *P. pseudodelicatissima* from the 1992 bloom did not produce detectable amounts of domoic acid (Lundholm *et al.*, 1994), although Martin *et al.* (1990) presented evidence to the contrary for a strain isolated from the Bay of Fundy, eastern Canada. The first confirmation of domoic acid production in a European *Pseudo-nitzschia* species was reported by Lundholm *et al.* (1994), who showed that some isolates of *P. seriata* from Danish coastal waters produced the toxin. Here, we report for the first time that a northwestern European isolate of *P. multiseries* is also able to produce domoic acid (Figs 19, 20). The amount of domoic acid produced by the *P. multiseries* strain W420Ppm5 isolated from the Dutch



Figs 13–18. Oligonucleotide probing of *Pseudo-nitzschia* spp. Fig. 13. *P. multiseries* (strain W420Ppm5) probed with *P. multiseries*-specific probe. Fig. 14. *P. multiseries* (strain W420Ppm5) probed with *P. pungens*-specific probe. Fig. 15. Light micrograph of cells shown in Fig. 14. Fig. 16. *P. pungens* (strain W420Ppp2) probed with *P. pungens*-specific probe. Fig. 17. *P. pungens* (strain W420Ppp2) probed with *P. multiseries*-specific probe. Fig. 18. Light micrograph of cells shown in Fig. 17. Scale bars represent 10 μm.

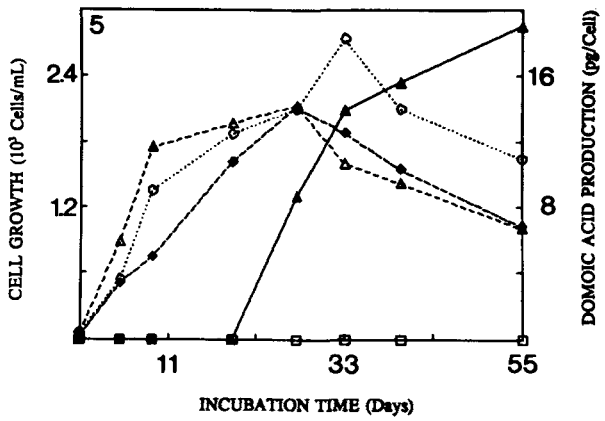


Fig. 19. Domoic acid production of *Pseudo-nitzschia* measured at 5–10 day intervals during growth. *P. multiseries* (△) CCMP1573 (as a positive control) and (◆) Wadden Sea isolate W420Ppm5, and (○) Wadden Sea isolate of *P. pungens* (as negative control). Production of domoic acid has been expressed as picograms per cell for *P. multiseries* strain W420Ppm5 (▲) and for *P. pungens* strain W420Ppm5 (□).

Wadden Sea (18·99 pg per cell at day 55), is comparable to quantities measured in North American isolates of this species (Bates *et al.*, 1989) and in *P. australis* (Buck *et al.*, 1992). Toxin was detected only after the stationary growth phase was reached, and not before. This result agrees with the findings of Bates *et al.* (1991) and Douglas & Bates (1992), who also found that domoic acid was not produced until stationary growth. On the other hand, the detection limit of our HPLC-UV method (detection limit 30·0 ng ml^{-1}) is less sensitive than that of the FMOC

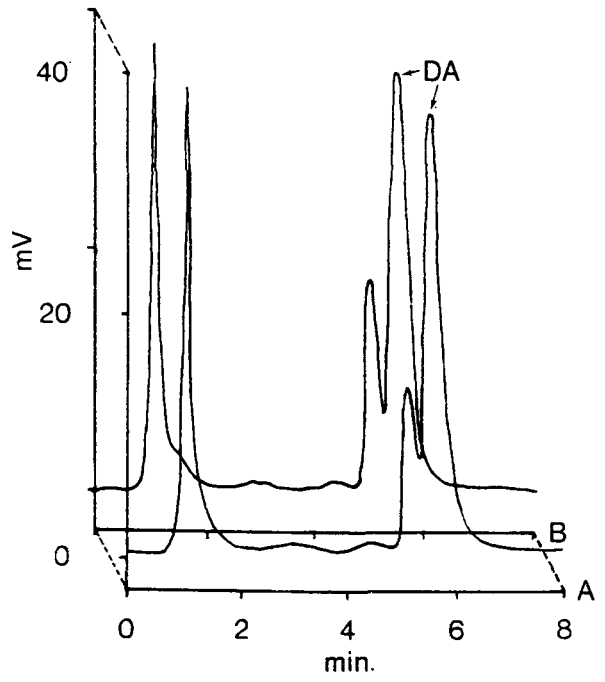


Fig. 20. HPLC-UV chromatogram of domoic acid (DA) analysis of a sample (day 55) of *P. multiseries* (W420Ppm5). Domoic acid has a retention time of 5·6 min. Partially spiked samples (A) were used to confirm the appearance of domoic acid in the crude sample (B).

method (detection limit 0·5 ng ml^{-1}; Pocklington *et al.*, 1990), and could result in false negatives at the beginning of the stationary growth phase. Nevertheless, the detection limit of the HPLC-UV method allows measurements below the critical value (20 μg domoic acid g^{-1} mussel tissue; Todd, 1993) for human toxicity. Therefore, warnings of the presence of domoic acid-contaminated shellfish can be issued when necessary. None of the isolates described here were axenic, and the presence of microorganisms may be implicated in toxin production. Production of domoic acid has been determined in axenic cultures of *P. multiseries* (Douglas & Bates, 1992; Douglas *et al.*, 1993), but the presence of bacteria increases domoic acid production 2- to 115-fold (Bates *et al.*, 1993).

The use of species-specific polyclonal antibodies (Bates *et al.*, 1993) allows examination of large numbers of samples, even when the concentration of target species is low. As reported by Bates *et al.* (1993) the two polyclonal antisera discriminate between toxin-producing and non-toxic forms of *Pseudo-nitzschia*. The labelling of Dutch Wadden Sea isolates was consistent with their findings (Figs 10–12): antibodies against the toxin-producer *P. multiseries* showed a clear reaction with the W420Ppm5 strain, while strains W30Ppp6, W420Ppp1 to 4, and W590Ppp1 to 7 reacted only with the antibodies against the non-toxic *P. pungens*. Weak cross-reaction between *P. fraudulenta* and *P. pseudodelicatissima* and the anti-*P. multiseries* antibodies was observed using a 1:50 dilution of the antiserum, as previously observed by Bates *et al.* (1993). Similarly, non-toxic *P. pungens* was clearly distinguished from the toxic *P. multiseries* using rRNA-targeted oligonucleotide probes for each organism (Figs 13–18). We therefore conclude that representatives of species from both North America and western Europe share common species-specific cell surface antigens and key rRNA signature sequences. More research is needed to examine rDNA sequences and restriction fragment length polymorphisms (RFLP) of LSU DNA (Scholin *et al.*, 1994) to confirm these findings. Results obtained to date suggest that western European strains of the toxic *P. multiseries* and the non-toxic *P. pungens* cannot be distinguished from North American isolates of the same species (C. A. Scholin *et al.*, unpublished).

In conclusion, *Pseudo-nitzschia* species have not yet been implicated in shellfish poisoning in Dutch coastal waters, but we have demonstrated that domoic acid is produced in a strain of *P. multiseries* isolated from the Wadden Sea. In order to reveal the precise distribution of this species we suggest the application of molecular probes both to stored survey samples and in future monitoring programmes. This molecular approach is needed because of numerous difficulties associated with the identification and enumeration of *P. pungens* and *P. multiseries* particularly when they co-occur in natural populations.

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